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SUFFIELD REPORT

NO. 435

ENZYME IMMUNOASSAY SYSTEMS UTILIZING POLYCLONAL ANTIBODY AS CAPTURE REAGENTS IN IDENTIFICATION OF NEWCASTLE DISEASE VIRUS ANTIGENS (U)

by

R.E. Fulton, N.P. Erhardt and R.I. Frank

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ABSTRACT

Four enzyme immunoassay systems, which utilize polyclonal antibodies as capture reagents attached to the wells of polystyrene microtiter plates, have been developed for the identification and quantitation of Newcastle Disease Virus, or its antigens, in environmental samples. Reagents were standardized and, using purified virus protein, the theoretical lower limits of sensitivity of each system was determined by dilution end-point. The enzyme immunoassay systems developed were 1000 to 2000 times more sensitive than the conventional hemagglutination assay and could detect virus protein at a concentration of 1 - 2 ng per well, or 3 - 9 ng per ml of test sample. Tests were simple to perform and, once capture antibodies had been adsorbed

to the solid phase, results could be obtained in 3 - 4 h. The techniques described are potentially adaptable to large scale automation for use in a laboratory setting or, alternatively, may be adapted for manual use in a field scenario.

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INTRODUCTION

Under the terms of reference of TTCP-E-TP4, Canada is responsible for the development of mammalian virus tracers (simulants) for field experimentation. These tracers are defined as live viruses which can be released into the open environment where they simulate biological attack by pathogenic viruses and are used to challenge the response of the collective protection and identification resources of the collaborating countries. In compliance with these requirements, Canada identified the La Sota vaccine strain of Newcastle Disease Virus (NDV) as the tracer of choice (Mofford and Fulton, 1982). Rapid and sensitive methods for identification of this virus or its antigens in environmen-

tal samples were then required. For this purpose, enzyme immunoassays, pioneered by Engvall and Perlmann, (1971) and Van Weemen and Schuurs, (1971), were considered to be the most appropriate of the available technologies for exploitation and development. Enzyme immunoassays are simple to perform, are highly sensitive and, unlike radioimmune assays, utilize reagents which are stable, present no health hazards and have long shelf-lives. Moreover, estimation of results can be made spectrophotometrically, or visually with the naked eye, thus permitting the option of portability into a field scenario. Enzyme immunoassays have been widely used for detection of antibodies to a variety of microbial and parasitic infections as well as to toxin detection and assays of hormones and a number of other chemical substances. Application of the method to virus identification is more limited. In this paper, we describe four enzyme immunoassay systems which utilize polyclonal antibody on the solid phase for capture, identification and quantitation of NDV antigen in environmental samples. All four systems are sensitive, simple and rapid to perform and may be adapted for use in the field. This communication constitutes the first published account of the use of enzyme immunoassay for the identification and quantitation of NDV.

MATERIALS AND METHODS

ANTISERA

1. Immunizations

Antisera to NDV were prepared by hyperimmunization of guinea pigs, chickens and mice, as follows.

a. Guinea Pigs

Female, albino guinea pigs, 350 - 400 g (Hartley outbred), were purchased from Charles River Ltd., St.Constant, Quebec. Purified NDV (protein concentration 100ug/ml) was mixed 1:1 with incomplete Freund's adjuvant and 1 ml of the resulting emulsion was injected intramuscularly (IM) into each of two sites (two thighs) of each guinea pig. Animals were subsequently boosted at two wk intervals for a period of 15 - 17 wk by injecting, IM, into each thigh, 0.5 ml of virus-adjuvant emulsion, prepared in the same manner as for the primary injection. Blood samples were taken by heart or choroid plexus puncture and the level of antibody response to NDV ascertained by hemagglutination inhibition (HI) test. To maximize the harvest of immune sera, animals were maintained for a total period of three months, during which time 10 - 25 ml samples of blood were withdrawn at approximate 10 day intervals. The resulting sera were pooled and stored at -60°C.

b. Chickens

Leghorn chickens (Shaver breed), 27 and 64 weeks of age, were purchased from La Poulet Poultry Farm, Medicine Hat, Alberta. Chickens were immunized with an initial dose of NDV vaccine (La Sota strain; Salsbury Laboratories, Kitchener, Ontario), administered in the drinking water, followed by a schedule of IM booster injections of purified virus given at weekly intervals. One liter of vaccine for drinking water administration was prepared as follows. One vial of lyophilized vaccine (1000 doses) was reconstituted in 30 ml of the diluent supplied by the manufacturer. Fifty doses (1.5 ml) of reconstituted vaccine were then added to 1 liter of distilled water containing 3 g of dissolved skim milk powder (Alpha brand). Drinking water was withheld from

the chickens for approximately 5 h prior to administration of the vaccine. The vaccine was then offered in a volume sufficient to supply approximately eight doses per chicken. Chickens were subsequently boosted at weekly intervals for a period of 12 - 15 wk by injecting, IM, into each of two sites (two thighs), 0.5 ml of an emulsion consisting of a 1:1 mixture of purified virus (100 μg/ml) and incomplete Freund's adjuvant. On the same day that booster injections were given, blood samples were withdrawn from the medial wing vein and the sera assayed by HI test for antibody titer to NDV. When the HI titer had reached a stable maximum, the volume of blood withdrawn from each chicken was increased to 30 - 50 ml at each bleeding session. At the end of 15 wk, serum samples were pooled and stored at -60°C.

c. Mice

Hyperimmune ascitic fluids in mice were prepared using a modification of methods described by Brandt et al., (1967), Russel et al., (1970) and Chiewsilp and McCown, (1972). Swiss Webster (white), female mice (COBS outbred), 6 - 8 wk of age, were purchased from Charles River Ltd. and immunized, as follows. On day one, each mouse was injected with 0.5 ml purified NDV (200 ug/ml) at three different sites: 0.2 ml intraperitoneally (IP), 0.2 ml subcutaneously (SC) in the center of the back, and 0.1 ml IM in the thigh. In addition, each mouse was administered 0.1 ml of complete Freund's adjuvant separately at the IP site. This schedule was repeated on day three with the exception that no adjuvant was given. A third and fourth series of injections of purified NDV (200 ug/ml) were given, IP, on days 24 and 30, respectively. In addition, on day 30 each mouse also received, IP, 0.5 ml of freshly harvested mouse sarcoma cells (S-180) (American Type Culture Collection, Rockville, Md.) at a concentration of 2×10^7 cells per ml.

On day 43, ascitic fluids were harvested by needle puncture of the peritoneum, clarified by centrifugation, and tested by HI for antibody titer to NDV. Fluids were pooled and stored at -60°C.

2. Purification

a. Precipitation of Immunoglobulins

Gamma globulin was isolated from guinea pig and chicken sera by five cycles of ammonium sulfate precipitation, at a final concentration of one-third saturation, according to established procedures (Campbell et al., 1970a). Purified gamma globulin was resuspended in one-half of the volume of the original serum aliquot and dialysed against normal saline over a period of 4 days. To confirm that dialysis was complete, a small sample of the dialysate was treated with barium chloride and examined for the presence of sulfate ions. The final product was evaluated for purity by immunoelectrophoresis and Ouchterlony gel diffusion.

Lipoproteins and immunoglobulins were precipitated from mouse ascitic fluids by a procedure described by Volk et al. (1982). For precipitation of lipoproteins, 1 ml of 5% sodium dextran sulfate was added to each 20 ml of ascitic fluid and the mixture incubated 1 h at 4°C. One ml of 11.1% calcium chloride was then added; the mixture was again maintained at 4°C for 1 h, followed by centrifugation at 10,000 x g for 60 min. Immunoglobulins were then precipitated from the supernatant fluid by the addition of saturated ammonium sulfate in a ratio of 1:1 (v/v), thus effecting one-half saturation. Precipitated immunoglobulins were collected by centrifugation and dissolved in water, followed by dialysis against phosphate buffered saline (PBS), pH 7.2. Purity of the final product was evaluated by immunoelectrophoresis.

b. Immunoelectrophoresis

Immunoelectrophoresis was performed using a modification of established techniques (Williams, 1971; Campbell et al., 1970b), as follows. A glass plate measuring approximately 10 cm x 10 cm was placed on a flat surface and a sheet of GelBond® support (FMC Corporation, Rockland, Maine), approximately 10 cm x 11 cm, was placed, hydrophilic side up, on top of the plate, in such a manner that approximately 1 cm of the GelBond support extended over the edge of the plate. A plastic U-shaped spacer, 1.5 mm. in thickness, was placed on top of the GelBond, followed by another 10 cm x 10 cm glass plate, and the whole "sandwich" clamped securely on three sides. A solution of 1% molten agarose in 0.025 M tricine buffer, pH 8.6, prepared by dissolving one agarose electrophoresis tablet (Bio-rad Laboratories, Mississauga, Ontario) in 5 ml distilled water, was then poured into the space between the plates where it was allowed to solidify for 30 min at 4°C. One of the glass plates and the plastic spacer were then gently removed, thus exposing the GelBond support sheet with its adherent covering of solidified agarose. Wells and troughs were cut in the gel and samples of each of the purified preparations of guinea pig, chicken and mouse immunoglobulins and of reference control whole guinea pig (Miles Scientific, Rexdale, Ontario), chicken (Jackson ImmunoResearch Laboratories, Avondale, Pa.), and mouse (Miles Scientific) sera were placed in appropriate wells. Electrophoresis was carried out in a model 1403 flat bed electrophoretic cell (Bio-Rad Laboratories) for a period of 1 h at 35 mA per gel. Troughs were filled with the appropriate anti-species whole serum (rabbit anti-guinea pig, rabbit anti-chicken, or rabbit anti-mouse whole serum) (Miles Scientific) or the corresponding anti-species IgG (rabbit anti-guinea pig, rabbit anti-chicken, or rabbit

anti-mouse IgG) (Miles Scientific) or the corresponding anti-species IgM (rabbit anti-guinea pig, goat anti-chicken, or rabbit anti-mouse IgM) (Miles Scientific). Diffusion was allowed to take place in a humid chamber at room temperature overnight. Unprecipitated protein was then removed from the gels by soaking them, under constant agitation, in a solution of 8.5% sodium chloride for a period of 1 - 2 days. To aid in visualization of precipitated proteins, gels were stained in a solution of comassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.): ethanol: acetic acid: distilled water; 1 g: 90 ml: 10 ml: 90 ml for 10 min. Gels were then destained in a solution of ethanol: glacial acetic acid: distilled water; 45 ml: 10 ml: 45 ml. Lipoproteins were stained for a period of 2 h in a solution of 0.1% Sudan Black B (Sigma Chemical Co.) in 60% ethanol, to which had been added, immediately prior to use, 0.06% (v/v) 30% sodium hydroxide. Gels were destained in 50% ethanol until backgrounds were clear (Uriel, 1971).

c. Ouchterlony Gel Diffusion

Ochterlony diffusion in gel was performed by conventional methodology (Campbell et al., 1970c), with the exception that gels were mounted on GelBond supports prior to staining. Briefly, the procedure was as follows. A solution of 1% molten agarose in 0.025 tricine buffer, pH 8.6, was prepared by dissolving one agarose electrophoresis tablet (Bio-Rad Laboratories) per 5 ml distilled water. Five ml of this solution was then poured into 50 x 9 mm Falcon Petri dishes (Fisher Scientific, Edmonton, Alberta); the lids were fitted tightly in place, and the agarose was allowed to solidify at 4°C for 30 min. Wells, 4 mm in diameter, were punched in the gels and each was filled with an appropriate serum preparation. Purified guinea pig immunoglobulin was reacted with rabbit anti-guinea pig whole

serum, rabbit anti-guinea pig IgG , and rabbit anti-guinea pig IgM (Miles Scientific). Similarly, purified chicken immunoglobulin was reacted with rabbit anti-chicken whole serum, rabbit anti-chicken IgG, goat anti-chicken IgM, and rabbit anti-chicken egg albumin (Miles Scientific). In addition, purified chicken and guinea pig immunoglobulins were reacted with each other. Plates were incubated in a moist environment overnight at room temperature. When diffusion was complete, unprecipitated proteins were removed by washing for several days in a solution of 8.5% sodium chloride. The gels were then transferred to GelBond supports and excess moisture removed by placing them, for a period of 1 -2 h, between a weighted "sandwich" of filter paper and paper toweling. The gels were then dried in an oven at 60°C for 30 min and stained in a solution of 0.5% comassie blue in ethanol:glacial acetic acid acid:distilled water; 45:10:45 (v/v). Gels were destained in a solution of ethanol:glacial acetic acid:distilled water; 45:10:45 (v/v).

d. IgG Fractionation and Affinity Purification

Further purification of guinea pig and chicken immunoglobulins was performed by contract by Jackson ImmunoResearch Laboratories. These procedures included fractionation and isolation of IgG by ion exchange chromatography and affinity immunoadsorption with the heterologous species. With respect to this latter step, in order to remove cross-reacting substances in each, detected by Ouchterlony gel diffusion and EIA, chicken IgG was immunoadsorbed with guinea pig IgG and guinea pig IgG with chicken IgG.

e. Peroxidase Conjugates

Purified guinea pig and chicken anti-NDV IgG,

respectively, were custom conjugated with horseradish peroxidase by Jackson ImmunoResearch Laboratories. Peroxidase-labelled affinity purified goat anti-chicken IgG (Kirkegaard and Perry) was purchased from Mandel Scientific (Edmonton, Alta.). Peroxidase-labelled rabbit anti-guinea pig and rabbit anti-mouse IgG were purchased from Miles Scientific.

VIRUS

1. Seed Stock and Propogation

NDV (La Sota strain) was purchased from American Type Culture Collection (Rockville, Maryland). The seed stock was cultivated, by established techniques (Hawkes,1979; Schmidt et al., 1965) in the allantoic cavity of embryonated hens' eggs. Briefly, fertilized eggs, purchased from Co-Op Hatchery (Calgary, Alberta), were incubated at 37 °C and 84% relative humidity and turned twice daily. On day 10 or 11, eggs were candled, the dead or infertile ones discarded, and the remaining fertile eggs inoculated with 0.1 ml of the appropriate dilution of virus. After inoculation, the puncture hole was painted with 70% alcohol, covered with a square of sterile aluminum foil, and sealed with cellulose tape. Eggs were incubated, "bandage"-side up, at 37°C and 84% relative humidity and candled daily for signs of embryo morbidity or death. When embryo death was observed or at a maximum of 5 days of incubation, the allantoic fluid was harvested. The fluid harvest from each egg was clarified by centrifugation at 850 g for 10 min and an aliquot checked for sterility by overnight incubation at 37°C in tryptose phosphate broth. In addition, the titer of each harvest was determined by hemagglutination test and those harvests which were sterile and were positive for hemagglutination were pooled and stored at -70°C.

2. Purification

a. Centrifugation

Purification of NDV from allantoic fluids was carried out in approximate 500 ml batches by a series of differential and rate zonal centrifugation steps. All differential centrifugation was carried out at 4°C in a Beckman model J2-21 preparative centrifuge (Beckman Instruments, Edmonton, Alberta). Rate zonal centrifugation was performed at 4°C in a Beckman model L8-70 ultracentrifuge (Beckman Instruments).

To remove cells and cellular debris, allantoic fluids were centrifuged for 10 min at 5350 x g and the resulting supernatant (supernatant 1), which contained the majority of virus, was set aside. The pellet, which contained intracellular virus, was resuspended in a small volume of 0.01 M monopotassium phosphate-disodium phosphate buffer, pH 6.7, held for 30 min at 4°C, homogenized in a Polytron homogenizer (Brinkman Instruments, Calgary, Alta.) for 1 min (speed 6), and centrifuged for 10 min at 5000 x g. The supernatant (supernatant 2) was pooled with supernatant 1 and the pellet was submitted to a repeat homogenization step. This process was repeated once in its entirety and the resulting three supernatants pooled. Pooled supernatants were then subjected to two cycles of differential centrifugation: 6400 x g for 15 min and 39,800 x g for 1 h.

The pelleted virus was resuspended in 0.01 M tris-HCl buffer, pH 7.4, containing 0.1 M sodium chloride and 1 mM ethylenediaminetetraacetic acid (EDTA) (STE buffer). An aliquot of this material was layered on a discontinuous sucrose gradient consisting of equal volumes of 60%, 40% and 20% (w/v) sucrose in STE buffer and then centrifuged at 113,000 x g for 4 h. The resulting visible bands were

collected from the top of the centrifuge tube by aspiration with a Pasteur pipette and each collected band was assayed for virus hemagglutinin by hemagglutination test and examined for the presence of virus by electron microscopy. The majority of virus was observed at the 20/40% sucrose interphase, a lesser amount at the 40/60% interphase, while minimal virus was present at the interphase of the buffer with 20% sucrose. Occasionally, when virus at the 20/40% sucrose interphase was inadequately separated from virus at the 40/60% interphase, the material at the two interphases and the 40% sucrose between the interphases were pooled and submitted to repeat centrifugation on another discontinuous gradient containing a disproportionately increased volume of 40% sucrose. Each of the separated bands was diluted with STE buffer and the virus pelleted by centrifugation for 1.5 h at 131,000 x g. Virus pellets from each of the bands were resuspended, separately, in STE buffer and layered on preformed continuous sucrose gradients, ranging in concentration from 17% to 53%, prepared using a Beckman density gradient former (Beckman Instruments). Gradients containing virus and control gradients without virus were centrifugated at 113,000 x g for 4 h and 1 ml fractions collected from an opening punctured in the bottom of the tube. The absorbance of each fraction of the virus-containing gradients was read in a model DU-8 spectrophotometer (Beckman Instruments) at 260 nm and 280 nm and the hemagglutinin titer of each determined by hemagglutination test. The refractive index of each fraction of the control gradient was read with a Bausch and Lomb refractometer (Fisher Scientific, Edmonton, Alberta), the percent sucrose determined, and this information used to evaluate gradient linearity. Fractions containing virus, as indicated by peaks in absorbance at 260 and 280 nm and confirmed by peaks in hemagglutination activity in corresponding fractions, were pooled and dialyzed against STE buffer.

The purity of pooled virus preparations was evaluated by polyacrylamide gel electrophoresis and by direct examination in a JEOL JEM-100CX transmission electron microscope.

b. Polyacrylamide Gel Electrophoresis

Purified NDV and the proteins from allantoic fluids obtained from 10 to 11 day-old uninfected control embryonated hens' eggs were simultaneously electrophoresed in both 9% and 11% SDS-polyacrylamide slab gels. The methodology for this procedure has been described elsewhere (Fulton and Shew, 1984).

Enzyme Immunoassay

a. Systems Evaluated

The enzyme immunoassay systems compared and evaluated are indicated (Table 1).

b. Standardization of Reagents

The optimal concentration of each capture antibody (CAb) was determined by titration with the appropriate homologous peroxidase-labelled anti-species IgG. Hence, guinea pig anti-NDV IgG was titrated with peroxidase-labelled rabbit anti-guinea pig IgG, chicken anti-NDV IgG with peroxidase-labelled goat anti-chicken IgG, and mouse anti-NDV IgG with peroxidase-labelled rabbit anti-mouse IgG (Miles Scientific). The optimal concentration of detector antibody (DAb) (indirect method) was determined by checkerboard titration with the optimal concentration of CAb, three different concentrations of antigen and, a fixed dilution (excess concentration) of indicator antibody (IAb). The optimal dilution of IAb was determined by

checkerboard titration with the optimal concentration of CAb, three different concentrations of antigen and, a fixed dilution (excess concentration) of indicator antibody (IAb). The optimal dilution of IAb was determined by checkerboard titration with the optimal concentrations of CAb (direct method) or, of CAb and DAb (indirect method), and three different concentrations of antigen. That dilution of IAb which yielded the highest ratio of positive to background (P/B) absorbance was selected as optimal.

c. EIA Procedure

Incubation times, temperatures, reagent concentrations and numerous other variables were investigated during the course of the development of these assays. The following conditions and procedures are those found to yield optimal results.

For the direct procedure, wells of flat-bottomed NUNC-Immuno 96 well microwell plates (Gibco Canada, Calgary, Alberta) were coated with 200 ul of CAb diluted in carbonate-bicarbonate buffer, pH 9.6. The plates were then covered and incubated overnight in a moist environment at 4 °C or were sealed with pressure sensitive acetate film and stored at 4°C until used. Immediately prior to use, the plates were washed. For this and all subsequent wash steps, the washing solution consisted of PBS, pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 (T) (PBS-BSA-T); washing cycles were repeated five times. The plates were tapped dry and unreacted sites blocked by the addition of 350 ul of PBS, containing 1% BSA and 0.05% T. After incubation for 1 h at 37°C, the blocking solution was removed and the plates were washed. Two hundred ul of purified NDV, diluted in PBS-BSA-T, was then added and the plates incubated for 2 h in a moist environment at

37°C. The plates were again washed. Two hundred ul of IAb, diluted in PBS-BSA-T, was added and the plates were incubated for 1 h at 37°C, then washed. Two hundred ul of Hybri-Clonal ® peroxidase substrate solution (ammonium 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate)) (ABTS) (Kirkegaard and Perry) (Mandell Scientific) was added and the plates incubated at room temperature for exactly 45 min. The color resulting from the hydrolysis of the substrate by the horseradish peroxidase bound in the previous steps was measured colorimetrically.

The indirect EIA was performed in the same manner as the direct procedure, with the following exceptions. After the washing step following incubation with the virus, 200 ul of DAb, diluted in PBS-BSA-T, was added and incubated for 1 h at 37°C. The plates were washed, then IAb was added and the plates incubated for 1 h at 37°C. The substrate solution (200 ul) was added and incubated at room temperature for 30 min.

d. Data Acquisition and Analysis

Absorbances of EIA reactions were read at a wavelength of 414 nm in a Titertek® Multiscan microplate colorimeter (Flow Laboratories) which was interfaced with an Apple IIe microcomputer. Data capture, formatting, storage and retrieval were achieved with a Titertek Multiscan data capture and storage software system (Drew Scientific, London, England). The colorimeter was blanked on an empty microwell plate and appropriate controls were included on each EIA test plate. Test reactions were considered to be positive if the mean absorbance in test wells was equal to or greater than twice the mean absorbance in negative control wells ie., P/B ratio equal to or greater than 2.

HA AND HI ASSAY

HA and HI tests were performed with 0.75% rooster erythrocytes (Institute Armand Frappier, Laval, Quebec) by standard techniques (Grist et al., 1974).

PROTEIN ESTIMATION

Protein concentrations of immunoglobulin preparations were estimated by a micro-Lowry procedure (Layne, 1957) or, by adsorption at 280 nm using a 1% extinction coefficient of 13.5 for chicken IgG and 14.0 for guinea pig IgG (Little and Donahue, 1968). Protein concentrations of purified virus preparations were determined by the micro-Lowry procedure or by absorbance measurements at 260 and 280 nm and application of the formula of Kalckar (Kalckar, 1947; Anonymous, 1974).

RESULTS

OPTIMIZATION OF CAB

The optimal concentration of each CAb was determined by titration with the appropriate peroxidase-labelled anti-species IgG. From the data obtained, curves relating CAb concentration and absorbance at 414 nm were constructed (Figs. 1, 2 and 3). For each of the three CAb titrated, absorbances increased with increasing additions of CAb up to a concentration maximum, beyond which further addition did not effect absorbance. The optimal concentration of CAb, defined as the lowest concentration yielding the maximum absorbance, was determined from each of these curves. The optimal concentration of guinea pig anti-NDV IgG CAb (Fig. 1) was 10 ug/ml; chicken and mouse anti-NDV IgG (Fig. 2 and 3, respectively) were optimal at 5 ug/ml.

OPTIMIZATION OF DAB

The optimal concentration of DAb (indirect EIA) was determined by coating wells with the optimal concentration of CAb followed by titration, in a checkerboard procedure, of DAb with varying concentrations of purified virus antigen and application of a fixed concentration of IAb. From the absorbances obtained, a curve relating concentration of DAb and antigen sensitivity was constructed (Fig. 4). With increasing concentrations of DAb, the minimum concentration of purified virus antigen detectable, expressed as the reciprocal of the antigen endpoint, increased up to a concentration maximum, after which further addition of DAb did not increase assay sensitivity. The optimal concentration of chicken anti-NDV IgG DAb was determined from this curve to be 5 ug/ml.

DETERMINATION OF ASSAY SENSITIVITY FOR ANTIGEN

The lowest concentration of virus antigen detectable by each of the EIA systems was determined by coating wells with the optimal concentration of CAb, followed by titration, in a checkerboard format, of purified virus protein with appropriate dilutions of the respective appropriate IAb. The antigen endpoint was defined as that concentration of virus protein detectable at an absorbance corresponding to twice the mean absorbance of the negative antigen control (twice background). This figure was derived algebraically by plotting the experimental data, generating the equation for the best fit linear curve[1] describing the data, and solving for concentration of antigen (x) by

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[1] $y = mx + b$, where m = slope and b = y intercept

substituting in the equation twice the mean background absorbance (y) (Figs. 5, 6, 7, and 8).

In each EIA system evaluated, antigen endpoints were tolerant of variation in dilution of IAb. Although actual absorbance values varied greatly within and between experiments when different dilutions of IAb were used, antigen endpoints obtained were similiar over a wide range of concentrations of IAb. The mean sensitivity of each EIA system was determined by pooling the antigen endpoints obtained utilizing various concentrations of IAb in experiments conducted in the same plates or on different plates, on the same day or on different days. Direct EIA systems utilizing guinea pig, chicken and mouse anti-NDV IgG CAb with, respectively, peroxidase-labelled chicken, guinea pig and guinea pig anti-NDV IgG IAb, and the indirect EIA system, were all sensitive to at least 9.3 ng/ml of purified virus protein (Table 2).

It was not possible, in spite of repeated trials, to obtain acceptably low antigen endpoints with the direct system utilizing mouse anti-NDV IgG CAb and peroxidase-labelled chicken anti-NDV IgG IAb. With this system, P/B absorbance ratios seldom exceeded two, hence, in these experiments, no antigen endpoint could be defined.

COMPARISON OF EIA TO HA FOR SENSITIVITY IN DETECTION OF ANTIGEN

Each of the EIA test systems developed was compared to the conventional HA test for sensitivity in detection of purified NDV antigen. To do this, the mean EIA endpoints expressed in ng/ml were converted to ng/well and the dilution of purified stock NDV required to achieve this endpoint calculated (Table 3). The mean HA titer (dilution endpoint) of the NDV stock was determined experimentally to be 1/6144. By assigning a sensitivity factor of 1 to the

reciprocal HA titer of the NDV stock, dilution endpoints of each of the EIA systems was compared to the dilution endpoint by HA. Accordingly, the EIA systems were 761 to 1927 times more sensitive than the HA test.

REPRODUCIBILITY

Well-to-well reproducibility was evaluated by adding 150 ul of IAb followed by 150 ul of ABTS to all wells of a battery of microwell plates and after one h reaction time, analyzing each for well-to-well variation in absorbance of the colored product. The mean and range of absorbances and coefficients of variation (CV) were calculated by row (designated 1 to 12) and by column (designated A to H). The mean CV by column (12%) (Range 10 - 14.7%) exceeded the mean CV by row (4%) (Range 2.9 - 5.5%). Examination of mean absorbances by row and column revealed that this discrepancy was accounted for by consistently higher absorbances in one row (row G). Whereas absorbances in rows A to F and in row H ranged from 0.10 to 0.12, those in row G ranged from 0.13 to 0.15. Subsequent to this finding, row G was not used as a test row, but was normally assigned for reagent control. Test controls were routinely assigned to the outermost rows and columns of each plate, while tests proper were carried out in the inner wells.

Reproducibility was also evaluated retrospectively from test results. Three levels of variation were examined: that occurring among replicate wells on a single plate on tests performed on the same day, that occurring among plates in experiments conducted on the same day and, that observed among antigen endpoints in experiments conducted on the same or different days (Table 4).

DISCUSSION

A number of methods for the identification and quantitation of antigen by EIA have been documented (Voller et al., 1980). The CAb method, which was selected for development in this study, has the advantage that samples may be assayed for antigen without purification; alternative methods require that antigen be purified either for attachment to the solid phase (inhibition method) or for enzyme-labelling (competetive method). The CAb method described in this study utilizes purified antibody on the solid phase to capture specific antigen; the presence of antigen is then indicated by reaction of enzyme-labelled anti-antigen antibody with its appropriate substrate (direct method) or is first detected with unlabelled anti-antigen antibody and indicated with enzyme-labelled anti-species antibody (indirect method).

EIA procedures adopted for routine use in this study were those which had been determined experimentally to be optimal. Other procedural variables investigated included length of time and temperature of incubation of CAb with the solid phase: 4°C for 3 days, 1 h or 3 h at 37°C followed by overnight at 4°C, overnight at 37°C; components of the blocking solution: 1 to 5% BSA, 1% human serum albumin, 1% gelatin, 1% guinea pig serum (with guinea pig CAb), 1% chicken serum (with chicken CAb); components of the washing solution: PBS, PBS containing 0.1% BSA, PBS containing 0.5% BSA and 0.05% T, PBS containing 0.05% T but no BSA; number of washes: ranging from 3 to 15 times and; length of time of incubation of DAb and of IAb: ranging from 15 to 60 min. For the sake of convenience and because prolonged adsorption procedures have been reported to result in more uniform coating (Anonymous, 1976), adsorption of CAb to the solid phase was routinely carried out at 4°C overnight.

Titration of CAb with the respective homologous peroxidase-labelled IAb generated curves which were biphasic, the initial slope being steep and with increasing concentrations of CAb, reaching a region of plateau at which further additions of CAb had no effect on the slope. Binding of CAb to the solid phase was thus a linear function of input up to a concentration maximum at which point the addition of higher concentrations did not result in further attachment. The attachment of proteins to plastic surfaces apparently occurs by hydrophobic interactions (Kochwa et al., 1967; Van Oss et al., 1966). In studies on the adsorptive characteristics of proteins for polystyrene, Cantarero et al., (1980), using radiolabelled proteins, showed that the amount of protein bound was dependent upon the concentration of protein added until saturation of the plastic surface occurred, at which point a constant amount of protein was bound regardless of the amount added. Furthermore, they showed that the proportion of protein bound is proportional to the amount added until the saturation point is reached, beyond which the proportion bound is inversely proportional to the amount added. Hence, it follows that no advantage results from the addition of CAb beyond its saturation concentration. In fact, it has been reported (Engvall and Ruoslahti, 1979) that high concentrations of protein during coating leads to increased desorption during incubation. The three immunoglobulins utilized in this study as capture reagents were optimized at concentrations ranging from 5 to 10 ug/ml. This finding is consistent with the published generalization which states that the optimum concentration of antibody for coating is between 1 - 10 ug/ml (Engvall and Ruoslahti, 1979). All proteins reach saturation at approximately the same concentration (Cantarero et al., 1980).

In the indirect EIA, titration of DAb in the presence of fixed concentrations of CAb and IAb, with varying

concentrations of antigen, yielded a biphasic curve similiar in shape to that observed for the titration of CAb. The concentration of DAb was directly related to assay sensitivity (lower limits of concentration of antigen detectable) and inversely related to the antigen endpoint. Hence, with increasing concentrations of DAb, the antigen endpoint decreased (assay sensitivity increased) up to a maximum point, after which, further addition of DAb did not increase the sensitivity of the assay. This relationship is not unexpected and can be accounted for by saturation of available antigen with DAb at the region of plateau.

Mean sensitivities of the EIA systems developed for the identification and quantitation of NDV antigen ranged from a high of 3.7 ng/ml (0.75 ng/well) to a low of 9.3 ng/ml (1.9 ng/well). The indirect method was as sensitive as the direct methods investigated and, although requiring one addition procedural step, offered the advantage that IAb could be purchased from commercial sources, thus obviating a requirement to label DAb in-house or by commercial contract. All of the EIA systems evaluated were tolerant of considerable variation in concentration of IAb. Within a wide range of dilutions, P/B ratios were essentially equivalent. This was advantageous in that day-to-day precision in preparation of IAb dilutions could be somewhat relaxed.

Antigen endpoints were determined by multiplying the mean absorbance of the antigen control by a factor of 2, substituting this value in the regression line equation describing the experimental data and solving for antigen endpoint, defined as that point on the x axis corresponding to the intersection of the regression and control curves. By the use of regression analysis and subsequent algebraic treatment of the data, antigen endpoints could be obtained objectively without operator-influenced interpretation of dose-response curves. That the data could validly be fitted

to straight line analysis was justified by the observation that a strictly linear relationship between antigen concentration and absorbance existed at the lower limits of antigen sensitivity.

A number of methods are currently used for detecting positive to negative cut-off values in EIA. These are (i) the threshold method, in which the cut-off value is assigned to the highest value of the negative reference control, (ii) the normal method, in which 2 or 3 standard deviations are added to the mean value of the negative reference control or, as was used in the present studies, (iii) the ratio method, in which the mean value of the negative reference control is multiplied by a factor of 1.8, 2 or 3 (Kramer et al., 1983; Herrmann et al., 1979; Voller et al., 1979). Since assay sensitivity is defined by the positive to negative cut-off level assigned, selection of this value is a significant element in assay design. In spite of this fact, no standard methodology for determination of positive to negative discrimination in EIA has been defined and selection of this value remains essentially arbitrary and without mathematical justifiction. (Kramer et al., 1983; Hick et al., 1980). This problem has recently received some attention and a statistical approach to definition of the cut-off value using discriminate function analysis of the data has been proposed (Kramer et al., 1983).

Variation within plates was evaluated initially in a single experiment by monitoring adsorbance readings on wells of plates treated with the same volume of the same reagents and retrospectively, for each of the EIA systems, on randomly selected replicate test wells in individual experiments conducted over time. On initial evaluation, the mean absorbance in one particular row was observed to be consistently higher than the mean absorbance of the other rows of the same plate. Position-dependent, within-plate variability among wells or blocks of wells of microtiter

plates used in EIA has been well documented (Denmark and Chessum, 1978; Halliday and Wisdom, 1978; Kricka et al., 1980). Properties of the plastics in the plate and thermal gradients have been suggested as contributing factors in this variation (Burt et al., 1979; Oliver et al., 1981; Sampson et al., 1985). Although systematic spacing of duplicates (Stemshorn et al., 1983), various placement designs (Burrows et al., 1984) and quality control of plate temperature (Sampson et al., 1985) have all been proposed as methods of controlling this problem, it is unlikely that any a priori standard arrangement of samples on a plate will be suitable for all plate types. This state of affairs thus necessitiates that each plate type be stringently evaluated for intrinsic patterns of variation prior to use in EIA and that reproducibility be routinely monitored over the course of experiments. In the retrospective evaluation, CV between replicate wells containing identical test reagents on the same plate or on different plates did not exceed 5.4% or 15.6%, respectively, in any of the EIA systems developed. Similarily, antigen endpoints obtained using the same batch of reagents and the same or different dilution of IAb, performed on the same or different days, did not exceed 17.1%. CV of these magnitudes (less than or equal to 20%) are considered to be small (Prepas, 1982) and imply that the relative variation between samples is not different.

Four microplate immunoassay systems (three direct and one indirect), which utilize polyclonal antibody attached to the solid phase as capture reagent, have been developed for the identification and quantitation of NDV antigens. By the ratio method of endpoint determination, the lower limits of sensitivity of these tests ranged from approximately 1 to 2 ng of virus protein per well or 3 to 9 ng per ml. This level of test sensitivity was determined to be approximately 1000 to 2000 times greater than that of the conventional HA

test for quantitation of NDV antigen. The EIA systems described are easy to perform and once CAb has been adsorbed to the solid phase, may be completed in 3 to 4 h. Readings can be made with a colorimeter, in which case results are quantitative, or may be estimated subjectively by eye. Although the former method elimates reader interpretation and bias and hence enhances accuracy, the latter method permits the flexibility of portability beyond the confines of a support laboratory. Tests can be easily automated for rapid processing of multiple samples in the laboratory, with computer analysis of results or alternatively, can be adapted for use in the field by relatively untrained and unskilled personnel. For field deployment of these assay systems, it is proposed to utilize a standard curve to estimate antigen concentration in a single sample dilution, thus obviating a requirement for multiple dilutions necessary to obtain an endpoint titer. The EIA systems described will be evaluated for their efficacy in identification and quantitation of NDV antigen in environmental samples, first by utilizing air samples which have been "spiked" with a known concentration of NDV. A full-scale field evaluation in which NDV will be aerosolized into and collected from the open air is scheduled to take place in the fall of 1986.

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TABLE 1. EIA SYSTEMS EVALUATED

DIRECT

CAb (anti-NDV IgG)	IAb (labelled anti-NDV IgG)
guinea pig	chicken
chicken	guinea pig
mouse	guinea pig
mouse	chicken

INDIRECT

CAb (anti-NDV IgG)	DAb (anti-NDV IgG)	IAb (labelled anti-species IgG)
mouse	chicken	goat anti-chicken

Abbreviations: CAb, capture antibody; DAb, detector antibody; IAb, indicator antibody

TABLE 2. SENSITIVITY OF EIA SYSTEMS FOR NDV ANTIGEN

			Sensitivity (ng/ml)		
			Mean	Range	SE
DIRECT					
CAb (anti-NDV IgG)	IAb (labelled anti-NDV IgG)				
guinea pig	chicken		9.3	7.2-10.9	0.59
chicken	guinea pig		3.7	1.7- 5.3	0.59
mouse	guinea pig		6.7	5.0- 8.9	0.47
INDIRECT					
CAb (anti-NDV IgG)	DAb (anti-NDV IgG)	IAb (labelled anti-species IgG)			
mouse	chicken	goat anti-chicken	6.9	6.1- 7.9	.32

Abbreviation: SE, standard error of the mean

TABLE 3. COMPARISON OF SENSITIVITIES OF EIA SYSTEMS AND HA ASSAY FOR NDV ANTIGEN

			Mean EIA Endpoint (ng/well)	Reciprocal Equivalent Dilution Endpoint[1]	HA Sensitivity Factor
DIRECT					
CAb (anti-NDV IgG)	IAb (labelled anti-NDV IgG)				
guinea pig	chicken		1.9	4,673,684	761
chicken	guinea pig		0.8	11,840,000	1,927
mouse	guinea pig		1.3	6,830,769	1,112
INDIRECT					
CAb (anti-NDV IgG)	DAb (anti-NDV IgG)	IAb (labelled anti-species IgG)			
mouse	chicken	goat anti-chicken	1.4	6,342,857	1,032

[1] Protein concentration of purified stock NDV was 8.88 mg/ml.

TABLE 4. REPRODUCIBILITY OF EIA SYSTEMS

			Mean Coeff. of Variation (%)		
			Well-to-Well	Plate-to-Plate	Antigen Endpoint
DIRECT					
CAb (anti-NDV IgG)		IAb (labelled anti-NDV IgG)			
guinea pig		chicken	4.9	4.8	15.6
chicken		guinea pig	4.8	5.8	17.1
mouse		guinea pig	4.2	15.6	16.6
INDIRECT					
CAb (anti-NDV IgG)	DAb (anti-NDV IgG)	IAb (labelled anti-species IgG)			
mouse	chicken	goat anti-chicken	5.4	ND	10.5

Abbreviation: ND, not done.

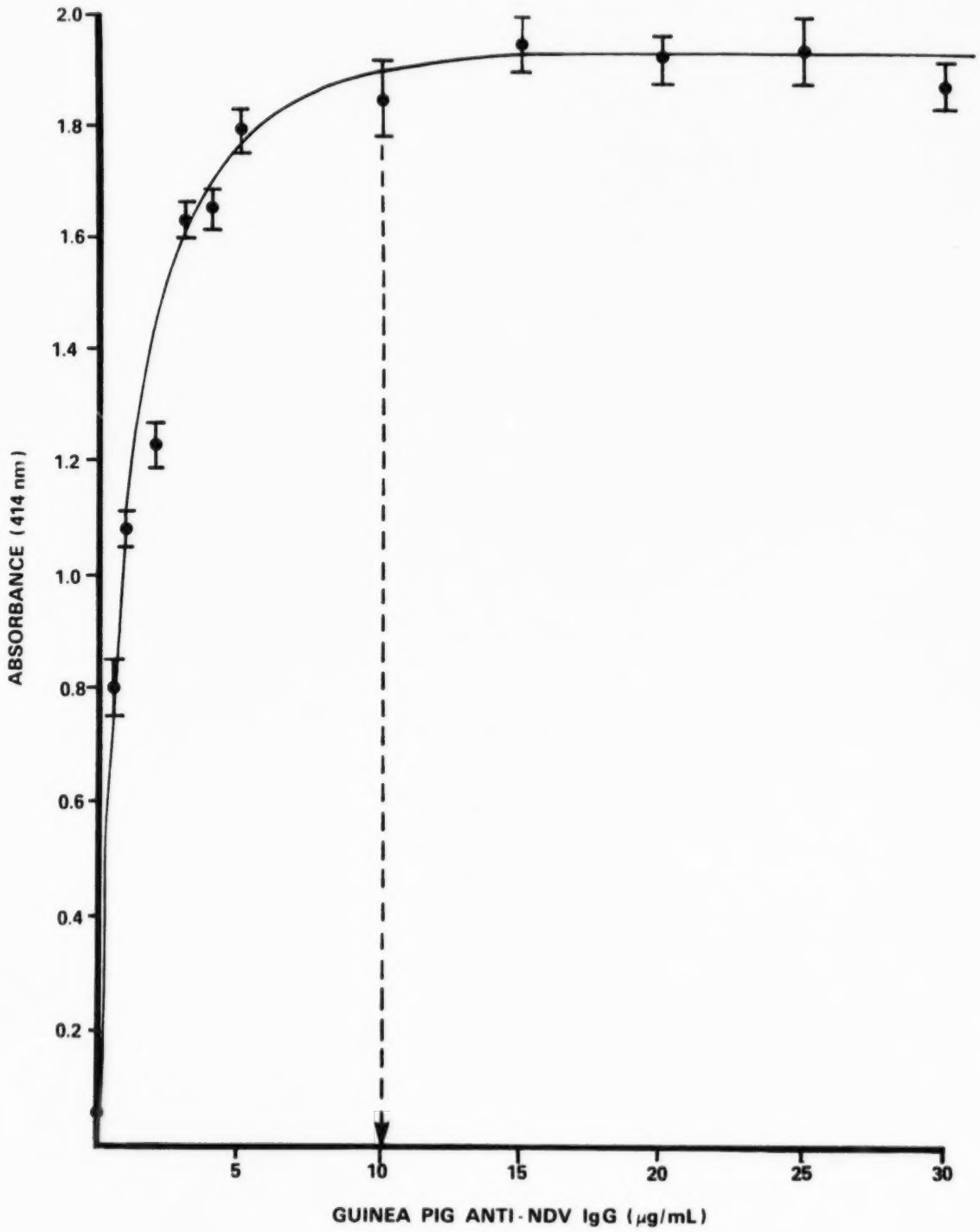

Figure 1

Optimization of guinea pig anti-NDV IgG CAb. Data points represent the mean of duplicate or quadruplicate determinations obtained on two separate days. Error bars represent the standard error of the mean. Arrow indicates optimal concentration of CAb.

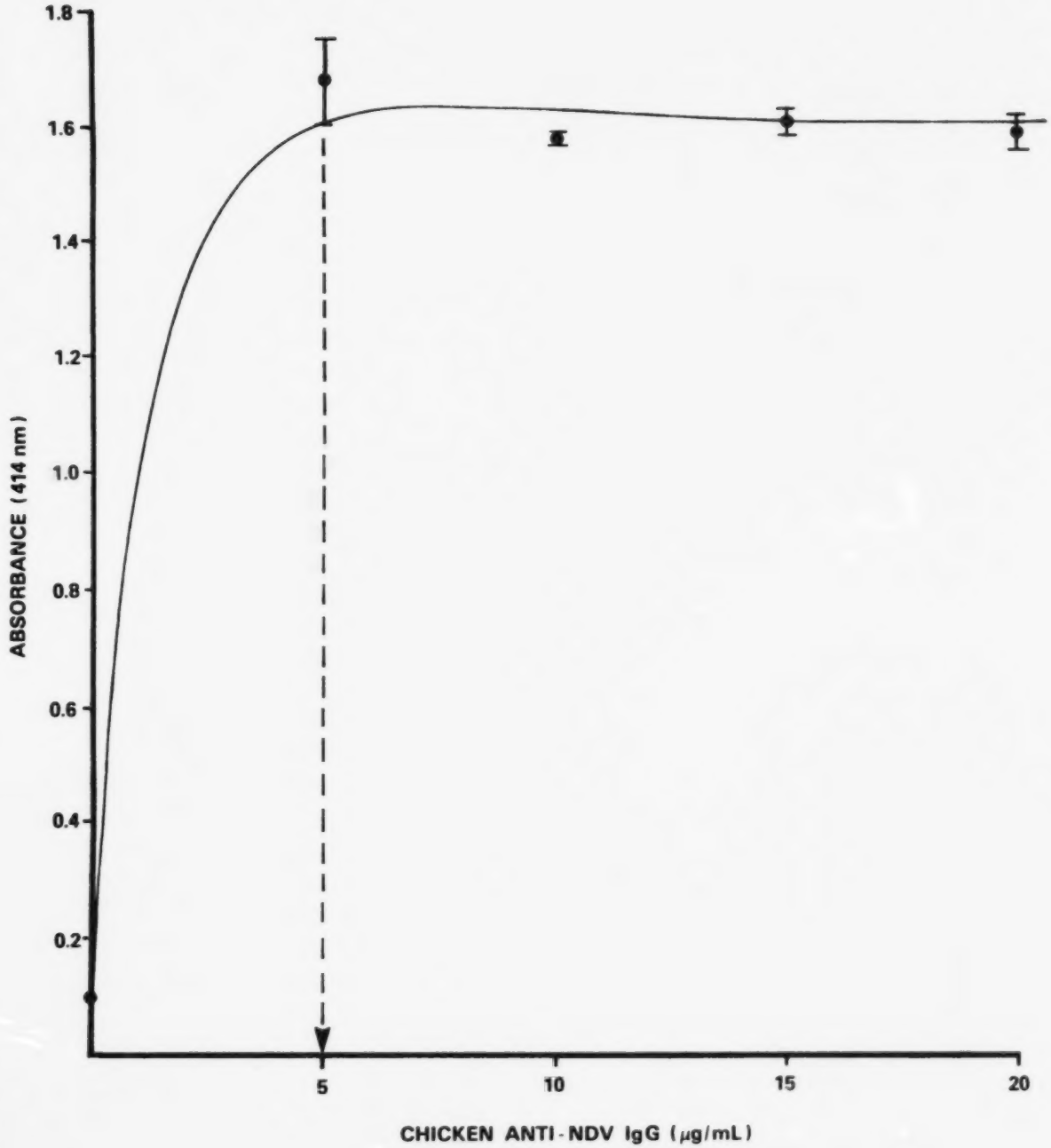

Figure 2

Optimization of chicken anti-NDV IgG CAb. Data points represent the mean of quadruplicate determinations on a single plate. Error bars represent the standard error of the mean. Arrow indicates optimal concentration of CAb.

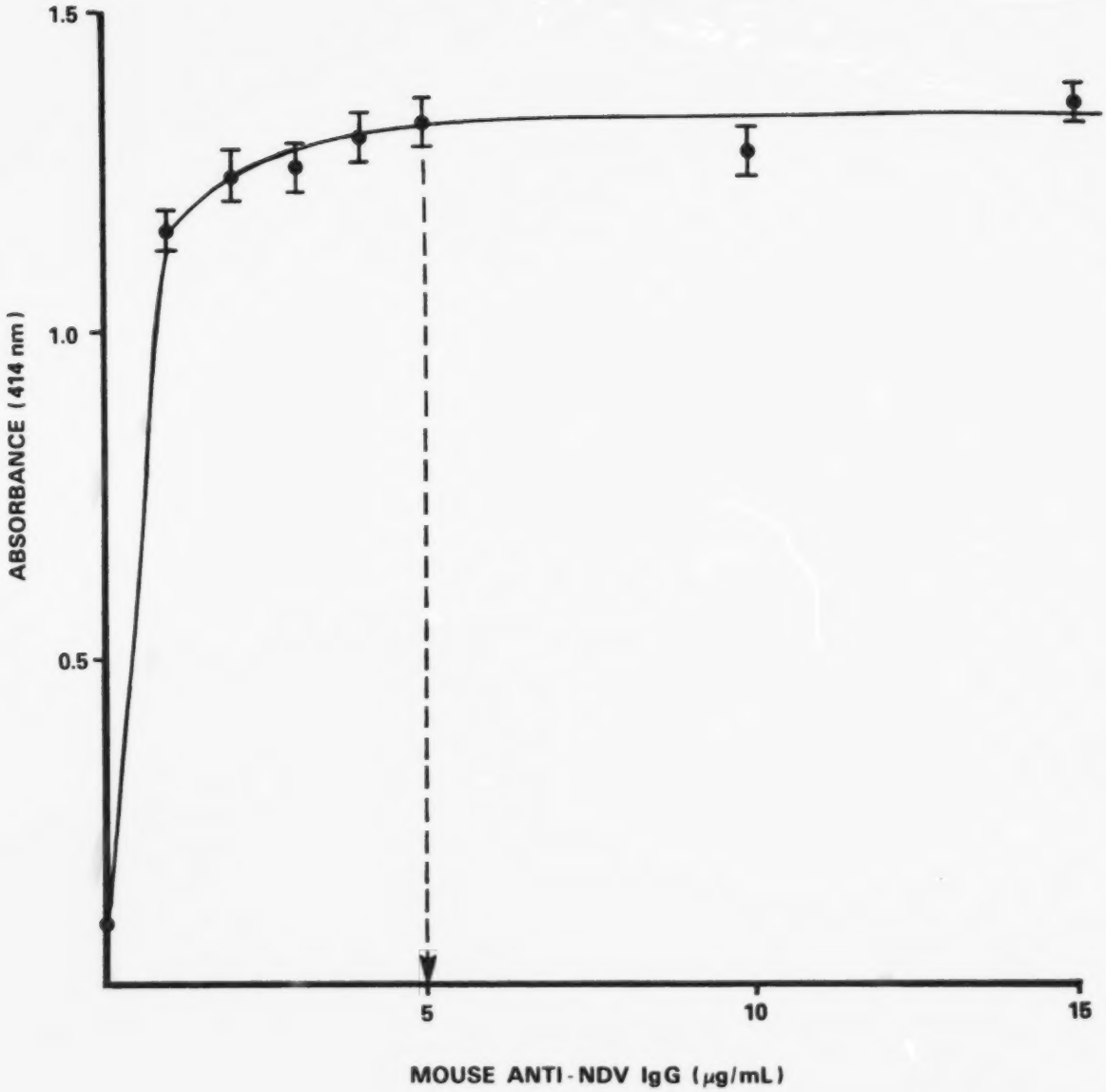

Figure 3

Optimization of mouse anti-NDV IgG CAb. Data points represent the mean of six determinations performed in duplicate on four separate plates and two separate days. Error bars represent the standard error of the mean. Arrow indicates optimal concentration of CAb.

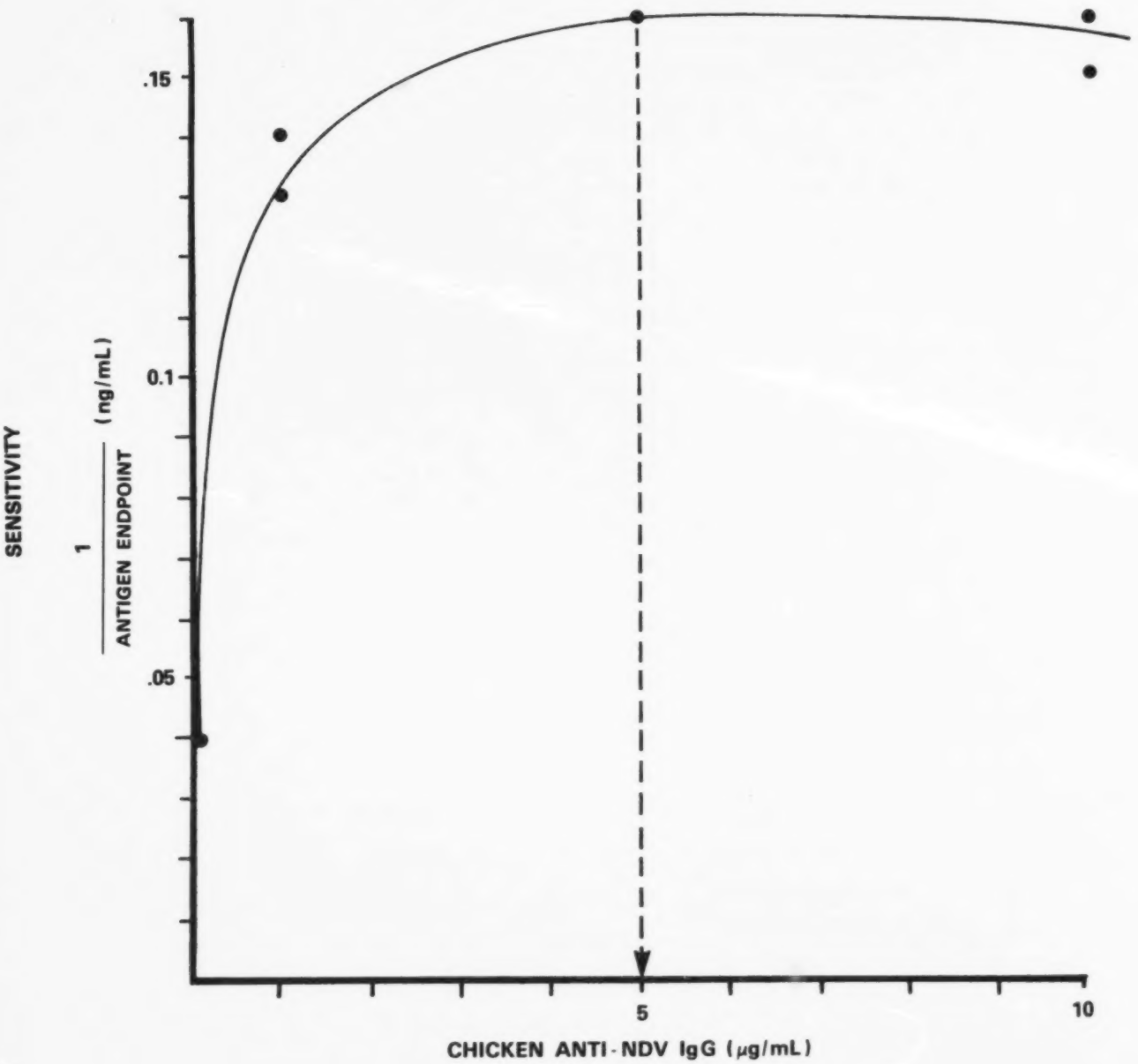

Figure 4

Effect of varying concentration of DAb on antigen endpoint in indirect EIA. Data points represent means of assays performed in quadruplicate on two separate days. Arrow indicates optimum concentration of DAb.

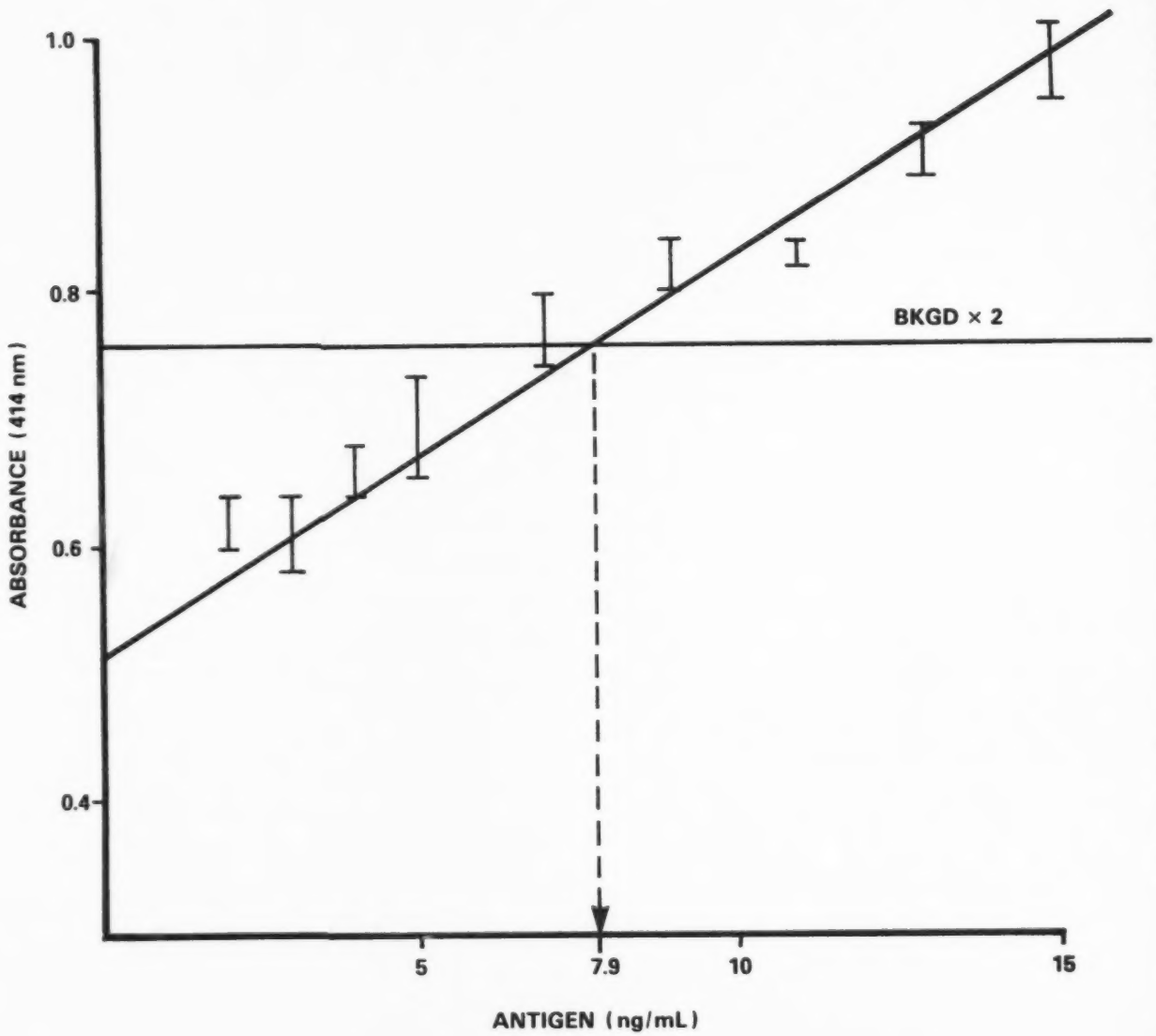

Figure 5

Antigen endpoint using guinea pig anti-NDV IgG CAb and peroxidase-labelled chicken anti-NDV IgG IAb. Regression line ($y = .515 + .031 \times$) was calculated from triplicate determinations on a single plate. Error bars represent standard error of the mean. Arrow indicates antigen endpoint.

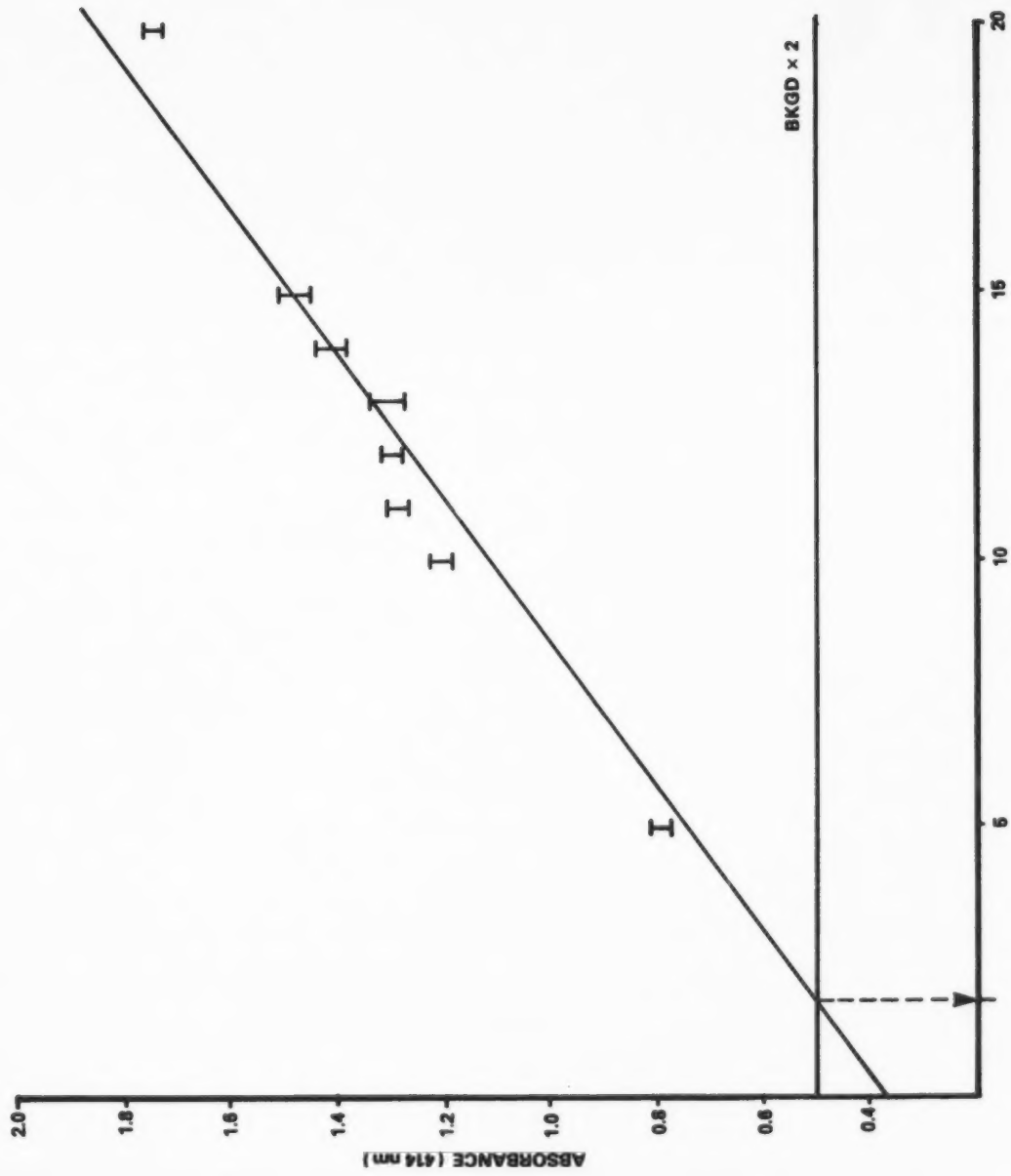

Figure 6

Antigen endpoint using chicken anti-NDV CAb and peroxidase-labelled guinea pig anti-NDV IgG IAb. Regression line ($y = .378 + .073x$) was calculated from five replicate determinations on a single plate. Error bars represent standard error of the mean. Arrow indicates antigen endpoint.

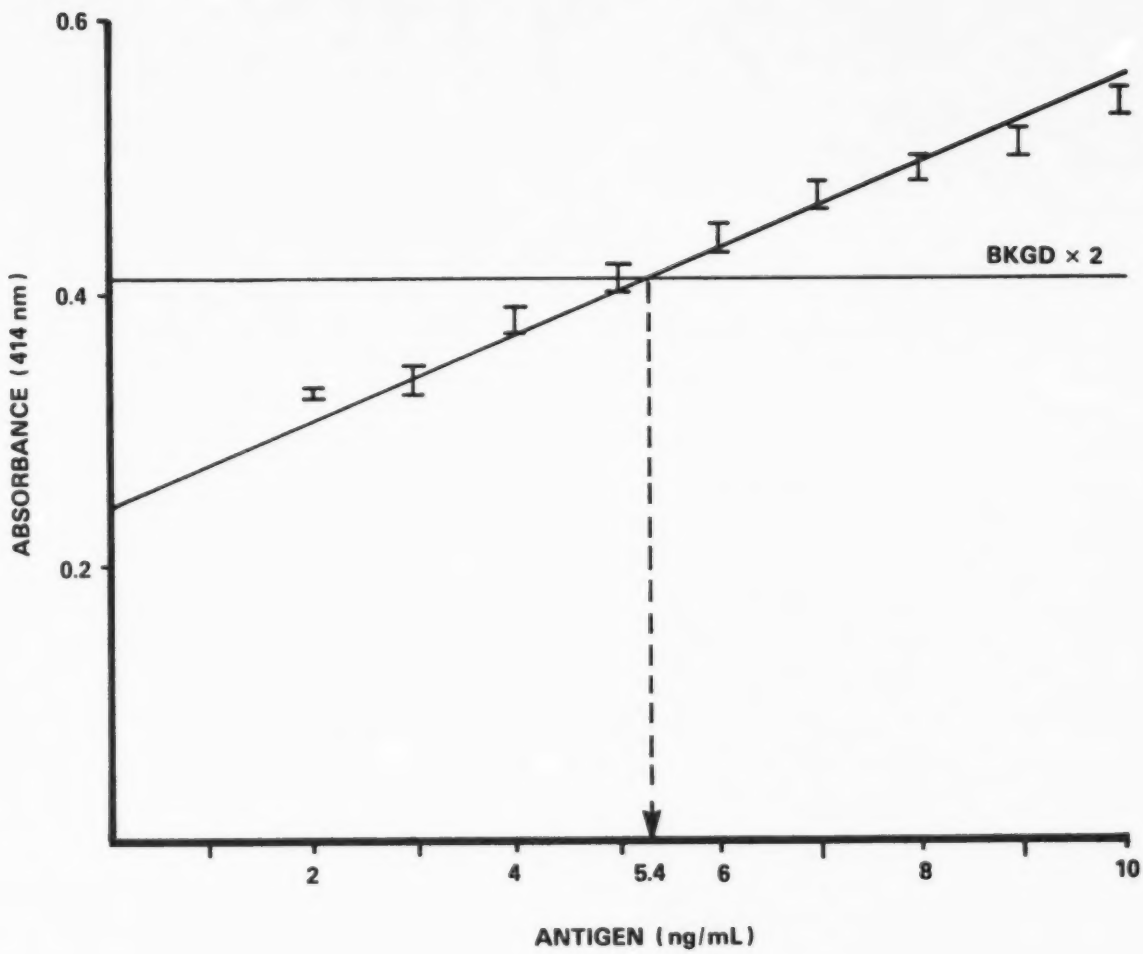

Figure 7

Antigen endpoint using mouse anti-NDV IgG CAb and peroxidase-labelled guinea pig anti-NDV IgG IAb. Regression line ($y = .237 + .032 \times$) was calculated from replicates of ten determinations on duplicate plates. Error bars represent standard error of the mean. Arrow indicates antigen endpoint.

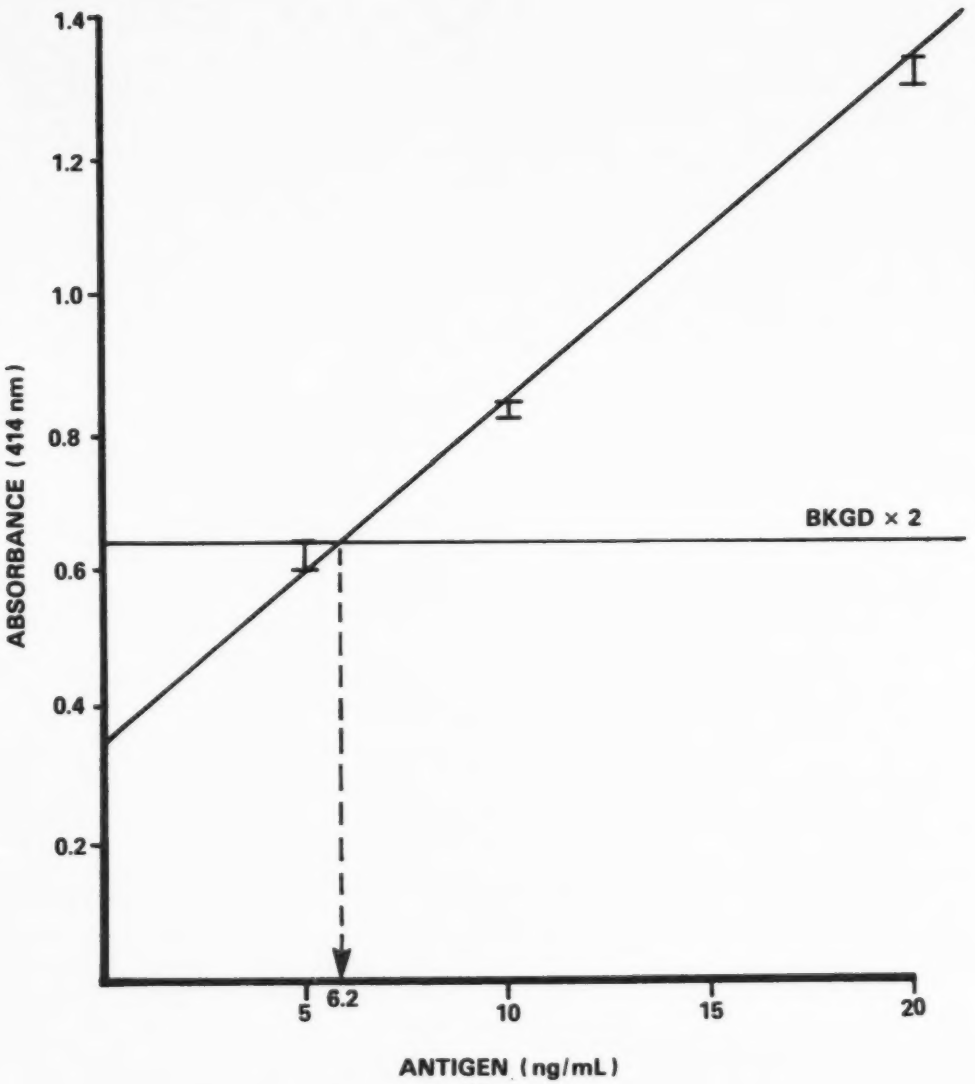

Figure 8

Antigen endpoint using indirect EIA. Mouse anti-NDV IgG CAb, chicken anti-NDV IgG DAb and peroxidase-labelled goat anti-chicken IgG IAb. Regression line was calculated from quadruplicate determinations on a single plate. Error bars represent standard error of the mean. Arrow indicates antigen endpoint.

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13. ABSTRACT

Four enzyme immunoassay systems, which utilize polyclonal antibodies as capture reagents attached to the wells of polystyrene microtiter plates, have been developed for the identification and quantitation of Newcastle Disease Virus, or its antigens, in environmental samples. Reagents were standardized and, using purified virus protein, the theoretical lower limits of sensitivity of each system was determined by dilution end-point. The enzyme immunoassay systems developed were 1000 to 2000 times more sensitive than the conventional hemagglutination assay and could detect virus protein at a concentration of 1 - 2 ng per well, or 3 - 9 ng per ml of test sample. Tests were simple to perform and, once capture antibodies had been adsorbed to the solid phase, results could be obtained in 3 - 4 h. The techniques described are potentially adaptable to large scale automation for use in a laboratory setting or, alternatively, may be adapted for manual use in a field scenario.

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